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# Defining Normal and Pathological Levels of Tumor-Associated Mutations


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**Abbreviations:**

ACB-PCR—allele-specific competitive blocker polymerase chain reaction
ACF—aberrant crypt foci
MF—mutant fraction


**Key Words**

Oncology biomarkers, cancer genetics, carcinogenesis, colorectal and anal cancer, oncogenes

Abstract: Allele-specific competitive blocker PCR (ACB-PCR) was developed at the U.S. Food and Drug Administration (FDA), National Center for Toxicological Research (NCTR), to quantify specific oncogene and tumor-suppressor gene mutations with high sensitivity. Using ACB-PCR, human colonic-mucosa tissues were shown to have a background or "normal" level of K-*RAS* mutation. Colon tumors were shown to have higher levels of K-*RAS* mutation than mucosa, even though the mutations are frequently present in tumors as subpopulations. K-*RAS* mutations were quantified in aberrant crypt foci and within the surrounding normal crypts. Because mutations in oncogenes, like K-*RAS,* have demonstrated prognostic significance, it would be useful to develop a quantitative understanding of normal and pathological levels of K-*RAS* and other tumor-associated mutations. Establishing databases of such measurements is needed as a foundation for using somatic mutations as oncology biomarkers in cancer-risk assessment and personalized medicine. This article represents an initial step toward that goal, as it includes individual K-*RAS* codon 12 GAT and GTT ACB-PCR measurements for human colonic tissue samples spanning the progression from normal colonic mucosa to colonic carcinoma.

## Introduction

Somatic mutations in oncogenes and tumor-suppressor genes are gaining importance in the practice of oncology and have potential applications in cancer screening. For example, detection of a K-*RAS* tumor mutation (as well as which particular K-RAS mutation is found) has prognostic significance for colorectal cancer patients [1]. Recently, it has been discovered that the K-*RAS* mutational status of advanced colorectal and lung tumors predicts patient response to biological therapies targeting the epidermal growth-factor receptor [1]. Further, a number of studies indicate that mutation in the *p53* gene has prognostic and therapeutic importance for several different cancers [2-4]. Clearly, progress has been made toward using somatic mutations as oncology biomarkers. However, the progress has been relatively modest given the central role such mutations are thought to play in carcinogenesis and the amount of resources and time invested in analyzing somatic oncogene and tumor-suppressor gene mutations as clinical biomarkers. To date, there are no implemented cancer-screening strategies based on the use of somatic mutations [1].

Why has the development of tumor-associated somatic mutations as cancer biomarkers been so intractable? It may be that carcinogenesis is a process too complicated to be evaluated based on single-molecular entities. Analyzing panels of mutations may be necessary. Such a strategy was employed in the PreGen-Plus test developed by Exact Sciences (Marlborough, Massachusetts) to screen for colorectal cancer. Analysis of multiple mutations improved the sensitivity of the test. Nevertheless, the sensitivity was deemed insufficient to justify the expense of the test [5, 6].

Another factor that may be impeding progress in developing tumor-associated mutations as oncology biomarkers is that they have been examined using inap-

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propriate tools and/or methodologies. Tumor-associated mutations have traditionally been analyzed by selecting a particular mutation-detection method and then determining whether particular mutations were present or absent in the clinical sample, as evaluated by that method. Because mutation-detection methods vary widely in sensitivity, this has led to conflicting reports in the literature regarding the presence of mutation(s) in different-tissue types and the frequency with which different mutations are detected in tumors [1]. Methods with greater sensitivity than DNA sequencing, which is commonly used to analyze tumor mutations, may reveal stronger correlations between particular mutations and particular tumor types.

Quantification of absolute levels of mutation is expected to lead to more consistent results between laboratories and more rapid progress in developing somatic mutations as oncology biomarkers. Quantification is essential for the use of most biomarkers. The concentrations of certain metabolites are measured to test for particular disease states, such as kidney or liver damage. Normal levels of metabolites are observed in healthy individuals, whereas disease is correlated with abnormal levels. Thus, the future utility of somatic mutations as oncology biomarkers is likely to depend on methods to quantify mutations, as well as data describing the levels of different mutations commonly found in normal and pathological tissues.

There are a number of sensitive techniques that provide mutant quantification. These include allele-specific competitive blocker PCR (ACB-PCR), constant denaturation capillary electrophoresis, the LigAmp assay, and real-time PCR with bead, emulsion, amplification, and magnetics [7-12]. In applying such methods to the development of somatic mutation as quantitative biomarkers, measurement of K-*RAS* mutation in colon cancer is an ideal starting point. K-*RAS* mutations occur predominantly at a few specific codons. K-*RAS* is one of the few oncogenes with established prognostic significance, and K-*RAS* mutation is known to be important in the etiology of colon cancer.

Using a semi-quantitative PCR restriction fragment-length-polymorphism method, Dieterle *et al.* [13] described levels of K-*RAS* mutation in colon tissues and tumors. Their assay, which had a reported sensitivity of $10^{-3}$, was used to categorize tumors and colonic mucosa as having K-*RAS* mutant to wild-type ratios of 0.1, 0.01, 0.001, or <0.001. They reported that 3/15 mucosa samples had a K-*RAS* mutant to wild-type ratio of $10^{-3}$ or greater. They found that 74/199 (38%) colon tumors were K-*RAS* positive. In addition, Dieterle *et al.* [13] showed that the ratio of K-*RAS* mutant to wild-type alleles in carcinomas varied over four orders of magnitude.

ACB-PCR is an allele-specific amplification method that uses a specific PCR-primer design to selectively amplify the mutant allele [14, 15]. ACB-PCR has a sensitivity of $10^{-5}$, meaning it can detect mutant allele in a 100,000-fold excess of wild-type allele. The ACB-PCR method is quantitative because levels of mutation in unknowns are analyzed in parallel with a set of standards with defined mutant fractions. This method was used to quantify K-*RAS* codon 12 GAT and GTT mutations within samples corresponding to various stages of colon carcinogenesis. These include normal mucosa from individuals without colon cancer, tumor-proximal mucosa samples (2-5 cm from a patient's tumor), tumor-distal mucosa samples (5 or more cm from a patient's tumor), aberrant-crypt foci (ACF), adenomas, and carcinomas. Specific methods, summary statistics for each tissue type, and statistical comparisons between tissue types (excluding ACF) have been published [16]. The current manuscript contains the raw ACB-PCR measurements of K-*RAS* codon 12 GAT and GTT mutant fraction (MF). Publication of these data constitutes an initial step toward creating a public database of somatic tumor-associated mutation frequencies for different muta-

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tions. Such information, when combined with that obtained from similar types of studies, can be used to establish the variability between individuals and aid in the interpretation of somatic mutations as quantitative biomarkers of cancer risk.

## Materials and Methods

This study was reviewed, approved, and conducted in accordance with the Federal Wide Assurance (FWA) filed by the FDA Research Involving Human Subjects Committee (RIHSC, FWA 00006196), the University of Arkansas for Medical Sciences Institutional Review Board (UAMS IRB, FWA 00001119 ), and the Central Arkansas Veterans Healthcare System IRB (CAVHS IRB#1, FWA 00006264). Subjects were recruited from CAVHS using inclusion and exclusion criteria and tissue collection procedures previously described [16]. Some tumor tissues and normal tissues from autopsy patients were obtained from the Cooperative Human Tissue Network. Colon tissues collected included, normal-appearing mucosa from subjects without colon cancer, normal-appearing tumor-proximal mucosa (mucosa 2 to 5 cm from a subject's tumor), normal-appearing tumor-distal mucosa (mucosa 5 or more cm from a subject's tumor), ACF, adenomas, and carcinomas.

Portions of some of the fresh tumor-distal mucosa samples were fixed with 2% paraformaldehyde for 1 hour at 4°C, stained with 0.2% methylene blue for 15 minutes, and examined under a dissection microscope for identification of ACF. Sections of tissue containing ACF were excised, dehydrated, paraffin embedded, and sectioned. Tissue curls were recovered from sections by manual dissection, using a needle under a dissecting microscope. Aberrant crypts of ACF, normal crypts adjacent to ACF, and normal crypts from noncontiguous sections of the same block were collected. Samples were rehydrated by sequential washes in xylene, a graded series of ethanol, and water (Nerl Reagent Grade Water, Nerl Diagnostic

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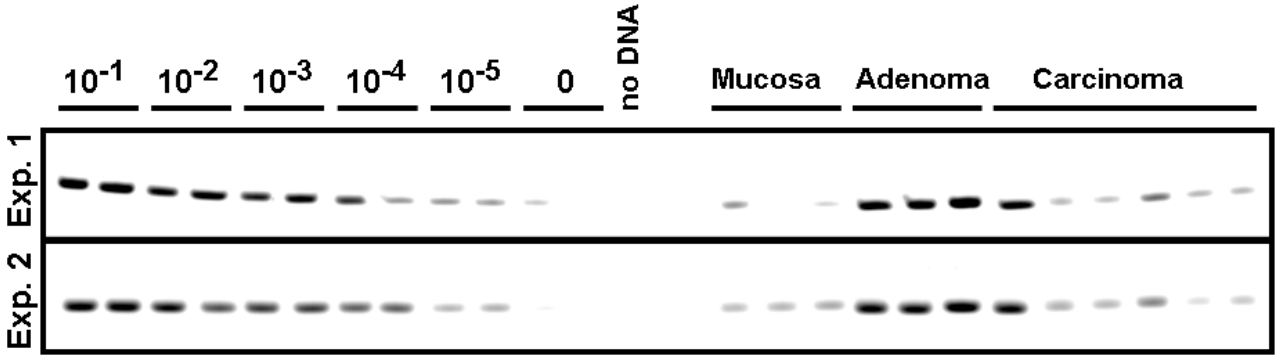


**Figure 1.** ACB-PCR measurement of K-*RAS* codon 12 GGT to GTT mutation. Two of the three replicate ACB-PCR results are shown. The MF standards are used to construct a standard curve (i.e., fit an exponential function) relating MF to ACB-PCR product-pixel intensity. Then the pixel intensity of the unknowns and the standard-curve function are used to calculate the K-*RAS* MF in each DNA sample.

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Corp., East Providence, Rhode Island). Thirty µL of proteinase K buffer were added to each sample, and the samples were incubated overnight at 60°C. Another 30 µL of 100 mM NaCl, 2.5 mM EDTA pH 8, and 0.1% SDS and 11.25 mL of Pefabloc solution were then added to each DNA sample. The Pefabloc solution was prepared by adding 1 mg of Pefabloc to 400 mL of 100 mM PIPES, pH 7.2, and 50 mL Pefabloc enhancer (Roche Applied Science, Indianapolis, Indiana). Samples were incubated for 1 h at room temperature, then 30 min at 37°C. Two µL of RNase solution were added, then the samples were incubated at 37°C for 2 h, and the DNA was ethanol precipitated using 2.5 M ammonium acetate. DNA was collected by centrifugation, re-suspended in 0.5X TE buffer, and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware).

A segment of the K-*RAS* gene was PCR amplified from the genomic DNA isolated from each individual tissue sample. The PCR products were gel-purified, quantified, and analyzed by ACB-PCR as previously described [16].

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**Table 1.** K-*RAS* codon 12 MF measurements of colonic mucosa.

| Mucosa Type | Subject ID | Subject Age | Colon Sample Location | Smoking History[a] | Codon 12 GAT Mutant Fraction | | | Codon 12 GTT Mutant Fraction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 2 | 3 | 1 | 2 | 3 |
| Normal (from subjects w/o colon tumor) | 7160-69 | 72 | Unknown | Unknown | 1.58E-04 | 1.16E-04 | 1.77E-04 | 1.40E-05 | 4.89E-06 | 1.51E-05 |
| | 7160-70 | 52 | Unknown | Unknown | 1.85E-05 | 3.81E-05 | 2.47E-05 | 1.18E-05 | 6.02E-06 | 9.81E-06 |
| | 7160-71 | 50 | Sigmoid | Unknown | 6.97E-05 | 1.25E-04 | 3.08E-04 | 3.01E-06 | 5.18E-06 | 2.64E-05 |
| | 7160-72 | 58 | Unknown | Unknown | 1.14E-04 | 9.63E-05 | 1.36E-04 | 1.94E-06 | 4.40E-06 | 1.37E-05 |
| | 7160-73 | 54 | Sigmoid | Unknown | 9.78E-04 | 2.38E-04 | 2.25E-04 | 2.76E-06 | 5.81E-06 | 2.53E-05 |
| | 7160-74 | 80 | Unknown | Unknown | 1.29E-04 | 5.28E-04 | 6.20E-05 | 1.21E-05 | 2.65E-05 | 3.97E-05 |
| Proximal (mucosa 2 – 5 cm from tumor) | 7160-2 | 57 | Sigmoid | 30 | 5.59E-04 | 1.90E-04 | 4.34E-04 | 1.00E-05 | 4.19E-06 | 8.38E-06 |
| | 7160-3 | 57 | Left | 0 | 2.83E-05 | 2.59E-05 | 2.52E-05 | 1.15E-05 | 5.38E-06 | 4.50E-06 |
| | 7160-4 | 58 | Right | 40 | 2.96E-05 | 3.14E-05 | 5.69E-06 | 8.82E-06 | 6.74E-06 | 6.57E-06 |
| | 7160-5 | 53 | Right | 30 | 1.24E-04 | 1.50E-04 | 6.51E-05 | 3.75E-06 | 4.32E-06 | 5.47E-06 |
| | 7160-6 | 52 | Right | 40 | 9.58E-05 | 8.46E-05 | 4.92E-05 | 2.85E-06 | 3.12E-06 | 5.04E-06 |
| | 7160-11 | 61 | Right | 0 | 1.09E-04 | 1.69E-04 | 1.06E-04 | 3.51E-06 | 1.00E-06 | 9.89E-06 |
| | 7160-12 | 63 | Right | 50 | 1.26E-04 | 8.63E-05 | 1.47E-04 | 1.79E-05 | 7.19E-06 | 1.56E-05 |
| | 7160-13 | 65 | Right | 67.5 | 1.05E-04 | 1.80E-04 | 1.96E-04 | 1.25E-05 | 6.89E-06 | 1.01E-05 |
| | 7160-14 | 64 | Left | 75 | 3.85E-05 | 2.42E-06 | 3.11E-05 | 9.03E-06 | 1.11E-05 | 1.15E-05 |
| | 7160-23 | 77 | Right | 20 | 6.97E-05 | 7.55E-05 | 4.07E-05 | 1.21E-05 | 8.60E-06 | 1.42E-05 |
| | 7160-24 | 77 | Right | 40 | 3.41E-05 | 1.76E-05 | 3.49E-05 | 5.72E-06 | 6.73E-06 | 5.89E-06 |
| | 7160-25 | 75 | Sigmoid | 60 | 3.12E-05 | 4.85E-05 | 3.48E-05 | 9.95E-06 | 1.00E-05 | 1.07E-05 |
| | 7160-26 | 70 | Sigmoid | 30 | 5.05E-04 | 1.30E-04 | 4.50E-05 | 3.11E-06 | 3.60E-06 | 5.61E-06 |
| | 7160-27 | 75 | Right | 50 | 6.64E-05 | 8.22E-05 | 6.98E-05 | 3.83E-06 | 3.51E-06 | 5.80E-06 |
| | 7160-28 | 73 | Right | 0 | 8.15E-05 | 2.93E-06 | 5.50E-05 | 3.08E-06 | 3.35E-06 | 5.70E-06 |
| | 7160-29 | 72 | Right | 75 | 4.15E-05 | 4.05E-05 | 4.59E-05 | 2.90E-06 | 2.78E-06 | 4.98E-06 |
| Distal (mucosa 5-10 cm from tumor) | 7160-4 | 58 | Right | 40 | 2.19E-05 | 2.42E-05 | 1.32E-05 | 8.24E-06 | 5.68E-06 | 4.52E-06 |
| | 7160-5 | 53 | Right | 30 | 5.15E-05 | 8.17E-04 | 3.33E-05 | 3.40E-06 | 3.49E-06 | 5.47E-06 |
| | 7160-6 | 52 | Right | 40 | 1.95E-04 | 1.64E-04 | 1.08E-04 | 3.12E-06 | 2.83E-06 | 5.37E-06 |
| | 7160-7 | 57 | Right | 10 | 2.78E-04 | 1.55E-04 | 5.17E-04 | 3.74E-06 | 1.08E-05 | 1.54E-05 |
| | 7160-11 | 61 | Right | 0 | 2.01E-04 | 1.35E-04 | 1.38E-04 | 1.62E-05 | 1.02E-05 | 1.25E-06 |
| | 7160-12 | 63 | Right | 50 | 1.38E-04 | 8.81E-05 | 9.02E-05 | 5.08E-06 | 4.75E-06 | 6.95E-06 |
| | 7160-14 | 64 | Left | 75 | 5.08E-05 | 3.83E-05 | 3.13E-05 | 6.20E-06 | 7.76E-06 | 9.01E-06 |
| | 7160-15 | 62 | Sigmoid | 80 | 9.65E-05 | 1.75E-04 | 2.74E-04 | 3.90E-06 | 6.88E-06 | 1.92E-05 |
| | 7160-22 | 72 | Sigmoid | 68 | 1.20E-04 | 4.67E-05 | 6.75E-05 | 3.98E-06 | 3.46E-06 | 8.44E-06 |
| | 7160-23 | 77 | Right | 20 | 1.05E-04 | 8.93E-05 | 4.74E-05 | 4.67E-06 | 2.52E-06 | 7.85E-06 |
| | 7160-25 | 77 | Right | 40 | 3.99E-05 | 6.15E-05 | 3.05E-05 | 7.49E-06 | 6.89E-06 | 5.11E-06 |
| | 7160-26 | 70 | Sigmoid | 30 | 4.27E-05 | 1.37E-04 | 1.53E-05 | 2.99E-06 | 2.93E-06 | 5.97E-06 |
| | 7160-27 | 75 | Right | 50 | 2.31E-04 | 4.09E-04 | 4.45E-05 | 2.62E-05 | 2.87E-05 | 4.03E-05 |
| | 7160-28 | 73 | Right | 0 | 7.90E-03 | 8.36E-03 | 3.92E-04 | 1.06E-05 | 1.06E-05 | 1.54E-05 |
| | 7160-29 | 72 | Right | 75 | 1.30E-04 | 2.62E-04 | 5.09E-05 | 2.36E-06 | 4.54E-06 | 6.92E-06 |

[a] When available, smoking history is reported in pack years.

**Table 2.** K-*RAS* codon 12 MF measurements of colonic tumors.

| Tumor Type | Subject ID | Subject Age | Colon Sample Location | Tumor Stage | Smoking History[a] | Codon 12 GAT Mutant Fraction | | | Codon 12 GTT Mutant Fraction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1 | 2 | 3 | 1 | 2 | 3 |
| Adenoma | 7160-11 | 61 | Right | | 0 | 1.96E-04 | 2.72E-04 | 9.54E-05 | 4.03E-01 | 1.40E+00 | 1.66E-01 |
| | 7160-16 | 60 | Right | | 20 | 1.85E-04 | 1.14E-04 | 1.56E-04 | 3.45E-06 | 2.77E-06 | 6.46E-06 |
| | 7160-22 | 72 | Sigmoid | | 68 | 6.38E-02 | 1.30E+00 | 2.43E-02 | 8.26E-03 | 1.85E-03 | 1.76E-03 |
| | 7160-24 | 77 | Sigmoid | | 40 | 2.93E-01 | 1.31E-01 | 2.73E-01 | 2.14E-02 | 1.20E-02 | 1.41E-03 |
| | 7160-27 | 75 | Right | | 50 | 4.57E-05 | 7.28E-05 | 4.01E-05 | 1.29E-05 | 7.58E-06 | 1.06E-05 |
| | 7160-29 | 72 | Right | | 75 | 1.86E-04 | 1.52E-04 | 2.01E-04 | 3.07E-06 | 2.78E-06 | 5.67E-06 |
| | 7160-46 | 66 | Right | | Unknown | 6.19E-02 | 4.69E-01 | 3.47E-02 | 9.74E-03 | 9.97E-04 | 8.80E-04 |
| | 7160-47 | 62 | Right | | Unknown | 1.60E-01 | 1.01E-01 | 1.14E-01 | 3.50E-03 | 3.72E-03 | 6.53E-04 |
| | 7160-48 | 66 | Right | | Unknown | 2.75E-03 | 4.31E-03 | 4.99E-03 | 3.36E-05 | 1.99E-05 | 1.52E-05 |
| | 7160-49 | 81 | Right | | Unknown | 1.16E-05 | 2.03E-05 | 2.04E-05 | 5.58E-01 | 9.58E-02 | 1.81E-02 |
| | 7160-50 | 72 | Sigmoid | | Unknown | 6.52E-04 | 2.80E-04 | 3.14E-04 | 4.66E-06 | 4.50E-06 | 6.12E-06 |
| | 7160-51 | 67 | Right | | Unknown | 1.60E-05 | 2.68E-05 | 3.79E-05 | 3.69E-03 | 1.62E-03 | 1.58E-03 |
| | 7160-52 | 65 | Right | | Unknown | 3.08E-04 | 5.01E-04 | 1.69E-04 | 1.61E-03 | 2.65E-03 | 3.35E-03 |
| | 7160-53 | 65 | Sigmoid | | Unknown | 6.80E-05 | 2.17E-05 | 1.76E-04 | 1.93E-02 | 8.72E-02 | 2.65E-02 |
| | 7160-54 | 78 | Right | | Unknown | 9.79E-05 | 3.36E-04 | 1.86E-04 | 1.16E-04 | 1.59E-04 | 2.49E-04 |
| Carcinoma | 7160-4 | 58 | Right | IIA | 40 | 3.88E-05 | 5.37E-05 | 3.47E-05 | 1.98E-06 | 1.33E-05 | 6.06E-06 |
| | 7160-5 | 53 | Right | IIA | 30 | 6.59E-03 | 6.26E-03 | 2.38E-03 | 4.15E-06 | 3.21E-06 | 6.23E-06 |
| | 7160-6 | 52 | Right | IIIA | 40 | 5.35E-03 | 1.75E-03 | 3.55E-03 | 3.67E-06 | 4.86E-06 | 9.53E-06 |
| | 7160-7 | 57 | Right | I | 10 | 4.54E-02 | 4.42E-02 | 5.78E-02 | 8.73E-06 | 6.05E-06 | 8.44E-06 |
| | 7160-12 | 63 | Right | IIIC | 50 | 4.11E-04 | 1.37E-03 | 2.21E-04 | 9.36E-06 | 6.38E-06 | 1.36E-05 |
| | 7160-14 | 64 | Left | I | 75 | 1.18E-04 | 2.41E-03 | 1.98E-03 | 1.02E-05 | 9.69E-06 | 3.02E-05 |
| | 7160-15 | 62 | Sigmoid | I | 80 | 7.03E-04 | 1.30E-03 | 6.93E-04 | 5.34E-06 | 4.31E-06 | 8.45E-06 |
| | 7160-23 | 77 | Right | IIIB | 20 | 9.41E-05 | 1.05E-04 | 2.19E-05 | 9.40E-07 | 4.86E-06 | 6.60E-06 |
| | 7160-24 | 77 | Right | I | 40 | 2.29E-05 | 5.42E-05 | 5.29E-05 | 7.13E-04 | 1.73E-03 | 1.87E-04 |
| | 7160-26 | 70 | Sigmoid | IIA | 75 | 7.49E-03 | 6.87E-03 | 7.90E-03 | 5.35E-06 | 3.83E-06 | 6.51E-06 |
| | 7160-28 | 73 | Right | IIIB | Unknown | 1.01E+00 | 4.06E-01 | 7.32E-01 | 1.54E-03 | 4.48E-04 | 1.25E-03 |
| | 7160-29 | 72 | Right | IIA | Unknown | 7.67E-05 | 1.10E-04 | 5.04E-05 | 4.07E-06 | 4.04E-06 | 7.51E-06 |
| | 7160-33 | 51 | Sigmoid | I | Unknown | 5.38E-01 | 5.08E+00 | 1.01E-01 | 2.05E-02 | 1.19E-03 | 7.03E-03 |
| | 7160-34 | 65 | Sigmoid | IV | Unknown | 1.24E-04 | 1.34E-04 | 3.87E-05 | 4.36E-06 | 6.66E-06 | 6.81E-06 |
| | 7160-35 | 57 | Left | IIA | Unknown | 6.67E-05 | 1.19E-04 | 4.02E-05 | 3.01E-05 | 2.62E-05 | 3.40E-05 |
| | 7160-36 | 64 | Right | IIA | Unknown | 5.41E-05 | 1.43E-04 | 2.62E-05 | 1.84E-06 | 3.04E-06 | 6.19E-06 |
| | 7160-37 | 51 | Sigmoid | IIIC | Unknown | 8.92E-06 | 7.54E-05 | 9.43E-05 | 7.85E-06 | 1.57E-05 | 4.84E-06 |
| | 7160-38 | 56 | Sigmoid | IV | Unknown | 7.06E-06 | 1.68E-05 | 1.88E-05 | 3.84E-06 | 7.13E-06 | 4.32E-06 |
| | 7160-39 | 77 | Right | IIA | Unknown | 2.01E-05 | 2.98E-05 | 2.53E-05 | 4.63E-06 | 1.26E-05 | 5.92E-06 |
| | 7160-40 | 60 | Right | IIA | Unknown | 3.90E-01 | 1.38E-01 | 3.38E-01 | 2.31E-03 | 7.02E-03 | 5.87E-04 |
| | 7160-41 | 57 | Transverse | IIIB | Unknown | 3.52E-01 | 1.35E-01 | 2.48E-01 | 2.18E-06 | 1.44E-02 | 5.21E-04 |
| | 7160-42 | 76 | Right | I | Unknown | 1.47E-04 | 4.53E-04 | 3.01E-04 | 1.76E-01 | 1.40E-01 | 2.89E-01 |

[a] When available, smoking history is reported in pack years.

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## Results

DNA fragments (encompassing K-*RAS* codon 12) were isolated following agarose-gel electrophoresis, and many single-use aliquots were prepared for each original DNA sample. Some of these aliquots were used for repeated DNA-concentration determination; other aliquots were used for ACB-PCR. Each first-round PCR product was analyzed in three independent ACB-PCR experiments. Each ACB-PCR experiment included the parallel analysis of a set of MF standards. The MF standards and unknowns were set up such that they contained equal numbers of the K-*RAS* DNA fragment (5 X $10^8$ molecules/ACB-PCR reaction). Duplicate MF standards were analyzed, which had mutant to wild-type ratios of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, or 0 (no-mutant control). Results of two replicate ACB-PCR experiments are shown in Figure 1. Because $10^{-5}$ is the lowest MF standard, $10^{-5}$ is considered the lower limit of accurate ACB-PCR quantitation.

The three independent ACB-PCR MF measurements for the K-*RAS* codon 12 GAT mutation,

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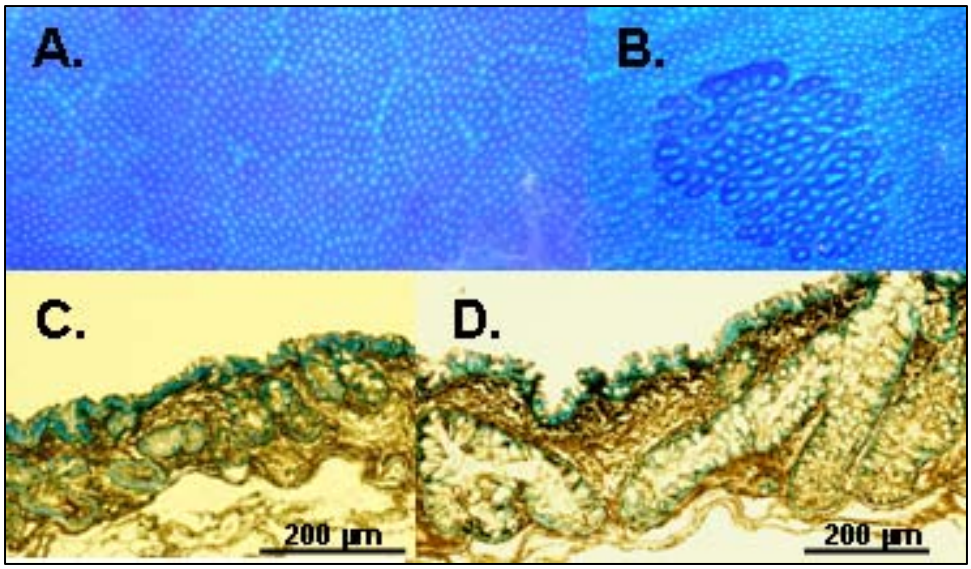


**Figure 2.** ACF identification and crypt characterization.
**A.** Normal colonic mucosa following paraformaldehyde fixation and methylene-blue staining
**B.** Aberrant crypt focus within the colonic mucosa
**C.** Representative normal crypts surrounding ACF in tissue sections
**D.** Representative example of aberrant crypts identified and then collected from ACF tissue sections

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and the codon 12 GTT mutation for all of the mucosa samples are given in Table 1. According to the numerical format used, 1.58E-04 indicates a MF of 1.58 x $10^{-4}$. The three independent ACB-PCR MF measurements of the K-*RAS* codon 12 GAT mutation, and the codon 12 GTT mutation for all of the tumor samples are given in Table 2. All but one of the carcinomas described in Table 2 were adenocarcinomas. The carcinoma from subject 7160-29 was a non-small cell carcinoma.

ACF were identified in the tumor-distal colonic mucosa of a single subject (7160-25). Figure 2 illustrates the normal structure of colonic mucosa, as compared to that containing an aberrant crypt focus. Normal colonic mucosa stained with methylene blue is shown in Figure 2A. An aberrant crypt focus is shown in Figure 2B. Sections of normal mucosa adjacent to sections of the aberrant crypt focus are shown in Figures 2C and 2D, respectively. Three independent ACB-PCR MF measurements for the K-*RAS* codon 12 GAT mutation and the codon 12 GTT mutation of ACF samples are given in Table 3. Each sample in Table 3 was collected from multiple adjacent-tissue sections. The normal crypt samples (Table 3) had significantly lower K-*RAS* MFs than did normal-appearing mucosa samples (Table 1). This indicates that the probability of detecting K-*RAS* mutation may be affected by sample size. When large-tissue samples are used for DNA isolation, then the ACB-PCR measurements represent the average across the tissue. When small-tissue samples are analyzed, there will be much larger variability between samples, and some samples may have measureable K-*RAS* while others may not.

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**Table 3.** K-*RAS* codon 12 MF measurements of normal crypts adjacent to ACF and aberrant crypts.

| Sample Type | Subject ID [a] (ACF #, Sample #) | Codon 12 GAT Mutant Fraction | | | Codon 12 GTT Mutant Fraction | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 |
| Normal Crypts (adjacent to ACF) | 7160-25 (F1, 1) | 2.89E-07 | 8.93E-07 | 8.27E-07 | 9.26E-07 | 2.53E-06 | 6.93E-06 |
| | 7160-25 (F1, 2) | 3.31E-07 | 9.58E-07 | 1.02E-06 | 9.99E-07 | 3.56E-06 | 8.02E-06 |
| | 7160-25 (F1, 3) | 3.89E-07 | 1.20E-06 | 1.28E-06 | 1.16E-06 | 3.09E-06 | 7.93E-06 |
| | 7160-25 (F1, 4) | 3.31E-07 | 1.14E-06 | 9.38E-07 | 1.20E-06 | 3.33E-06 | 8.18E-06 |
| | 7160-25 (F2, 1) | 7.28E-05 | 1.38E-03 | 2.47E-04 | 9.51E-07 | 3.90E-06 | 8.72E-06 |
| | 7160-25 (F4, 1) | 3.74E-06 | 5.69E-06 | 2.61E-06 | 1.75E-06 | 4.68E-06 | 9.44E-06 |
| | 7160-25 (F4, 2) | 1.48E-06 | 1.56E-05 | 5.16E-06 | 1.31E-06 | 4.19E-06 | 8.43E-06 |
| ACF | 7160-25 (F1) | 5.87E-07 | 1.18E-06 | 1.22E-06 | 1.03E-06 | 3.12E-06 | 8.40E-06 |
| | 7160-25 (F2, 1) | 2.84E-02 | 2.07E-02 | 3.89E-02 | 2.62E-02 | 1.74E-02 | 5.83E-02 |
| | 7160-25 (F2, 2) | 2.86E-03 | 3.35E-02 | 1.99E-02 | 2.26E-05 | 1.14E-04 | 2.09E-04 |
| | 7160-25 (F4) | 3.65E-07 | 1.56E-06 | 1.26E-06 | 1.07E-06 | 3.14E-06 | 8.60E-06 |

[a] All samples were from the sigmoid colon of a 75-year-old with a smoking history of 60-pack years.

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## Conclusion

In conclusion, progress toward using somatic oncomutations as cancer biomarkers will require quantification of oncomutation, as well as collection and curation of this type of data.

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## Glossary

Aberrant crypt foci—Clusters of abnormal crypts in the lining of the colon. One of the earliest, histological changes observed in the colon that may lead to the development of cancer (pre-neoplastic lesions).

Carcinogenesis—The process by which normal cells are converted into cancer cells.

Oncogene—A mutated (altered) form of a gene (proto-oncogene) involved in normal cell growth or homeostasis. When an oncogene is mutated, or expressed at high levels, it promotes conversion of normal cells into a tumor cell.

Oncology biomarker—Biological molecules found in blood, body fluids, or tissues that have significance regarding the probability of occurrence, diagnosis, prognosis, or treatment of cancer.

Somatic mutation—An alternation of DNA that occurs in any cell of the body (other than germ cells) and which may have the potential to cause cancer.

Tumor-associated mutations—Mutations frequently observed or detected in tumors.

Tumor suppressor gene—A gene whose function normally prevents or controls the growth of cancer cells, often through regulation of cell division, regulation of cell death, or by repairing damaged DNA. Mutations in tumor-suppressor genes, therefore, can promote tumor development.

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Photo/Danny Tucker

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menting and validating genomic techniques utilizing laser-capture micro dissection to search for biomarkers of neurotoxicity.

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## RRP's Research Spotlight

### A Newly Emerged Field of Epigenetics Opens New Horizons

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Until recently our understanding of stable heritable phenotypes were limited to a genetic paradigm based on the heritable stability of the DNA sequence. However, not everything could be explained by genetics, and what we saw in the DNA sequence became just the tip of the iceberg. The real iceberg body existed under the surface,

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## RRP's Research Spotlight

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invisible and underestimated, and has remained *terra incognita* until the end of the last century. Since then, epigenetics has rapidly emerged as a key player in the field of modern biology, receiving more and more attention from researchers. The Latin prefix "epi" means *above* or *over*. Indeed, "epigenetics" is defined as heritable changes in gene expression associated with modifications of DNA or chromatin proteins that are not due to any alteration in the primary DNA sequence. Such modifications include the best known and much studied methylation of DNA (i.e., covalent addition of a methyl group [-CH3] to the cytosine residue at CpG DNA sequences) and posttranslational modifications (e.g., methylation, acetylation, and phosphorylation) of the proteins that bind to DNA (histones). These epigenetic modifications (controls) are heritable and are essential for the normal development and proper maintenance of cellular functions in adult organisms. It is widely accepted that such important biological processes as X-chromosome silencing, imprinting, silencing of foreign repetitive elements, accurate regulation of gene functioning, maintenance of the proper chromatin structure, DNA repair, and many others are solely under the control of epigenetic mechanisms.

However, like most biological events, epigenetic control can become dysregulated and lead to the development of a wide-range of human pathologies. Recent research links dysregulation of epigenetic machinery with cardiovascular diseases; neurodegenerative and psychiatric disorders, such as Alzheimer disease and schizophrenia; autoimmune diseases; and cancer. With the exploration of the field of epigenetics today, we are closer to understanding the mechanisms that underlie development and progression of disease. For example, loss of global genomic methylation paralleled by promoter hypermethylation (increased methylation) of selective tumor-suppressor genes is a well-known feature of many human cancers. Additionally, it has been proposed that epigenetic alterations, including genomic and repeat-associated hypomethylation (decreased methylation), may precede and/or provoke genetic alterations. With that in mind, epigenetics might provide us with a set of specific biological markers that would aid in early detection of many pathological states. More importantly, considering that a remarkable feature of epigenetic abnormalities is their potential reversibility, timely correction and accurate maintenance of the cellular epigenetic status are promising avenues to make treatment and preventive modalities more effective. Indeed, several drugs that target epigenetic mechanisms, such as the histone deacetylase inhibitor Vorinostat (Zolinza) and two DNA demethylating agents, Vidaza and Decitabine, have successfully passed clinical trials and have been approved by the U.S. Food and Drug Administration for treatment of myelodysplastic syndrome.

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In summary, epigenetic alterations are crucial determinants of many human diseases. Understanding the mechanisms associated with epigenetic dysregulation and its correction will provide a unique opportunity to use them as early diagnostic biomarkers and opens a novel mechanism-based approach for disease prevention and treatment.

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## Editorial Matters

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